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Applicability and limitations of multiparametric fluorescence measurements to assess pigment concentrations in leafy vegetables

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1. Introduction, Knowledge, Objectives

There is growing interest to monitor plant stress responses, developmental processes and concentrations of health-promoting pigments in vegetable crops non-invasively. Different sensors estimate plant pigment content as indicators for plant performance and beneficial properties of harvested vegetable products. The portable fluorescence sensor Multiplex® (Force-A, Orsay Cedex, France) assesses plant pigment content by their screening effect on chlorophyll fluorescence. Multiparametric measurements upon excitation and detection of light with different wavelengths are used to calculate vegetation indices that quantify for plant pigment concentrations (Ben Ghozlen et al., 2010). This method is increasingly used to monitor stress-related changes in plant pigments and flavonoids in horticultural experiments (e.g. Kautz et al., 2014), but there is little evidence for an exact quantification of these leaf compounds.

In this study, we tested if measurements by Multiplex® are useful for assessing anthocyanin, flavonoid and chlorophyll concentrations in the leaves of three vegetable crops: red cabbage, spinach and tomato. These crops were subjected to N deficiency treatments, which are known to lead to reduced chlorophyll content and increased accumulation of flavonoids and anthocyanins in leaves. To demonstrate the applicability of the Multiplex® to monitor plant stress responses, multiparametric measurements as well as biochemical analyses of leaves of well-fertilized and N-deficient plants were performed and indices for chlorophyll, anthocyanin and flavonoid content were correlated to the respective biochemical analyses of leaf samples.

2. Material and Methods

Tomato plants `Lyterno´ (Rijk Zwaan, Netherlands) were grown in rockwool substrate in climate chambers (temperature of 22/18 °C, 50% relative humidity, 200 µE light intensity for 10 h per day) and fertilized with Hoagland nutrient solution. One-month-old plants were subjected to a one week control or N deficiency treatment with fertilization with full strength or N-free Hoagland solution. 0, 2, 4, and 7 days after start of the treatment, the fourth oldest leaf of n=3 plants was detached, immediately measured using a Multiplex® and sampled.

Red cabbage `Lodero´ (Bejo Zaden B.V., Warmenhuizen, Netherlands) and spinach `Silverwhale´ (Rijk Zwaan Welver GmbH, Welver, Germany) were grown in the field in 2012 and early summer 2013, respectively. Half of the plants of each vegetable crop were either fertilized with N according to the recommendations or with 50% of these recommended levels (see Schmidt and Zinkernagel, 2015). Upon harvests (66 days after sowing for spinach, 183 days after sowing for red cabbage), randomly chosen samples were taken from the marketable vegetables for Multiplex® measurements and chemical analyses.

Multiplex® (Force-A, France) measurements were taken on one leaf per plant. For tomato plants, a Multiplex® 3 was used (n=3, 5 technical replicates). For spinach and red cabbage, a Multiplex® Research was used (n=16 and n=12, respectively, with 3 technical replicates each). From every measurement, the Multiplex® indices FLAV, SFR_R and ANTH_RG for estimation of flavonoids, chlorophylls and anthocyanins were obtained. The measured leaves were immediately sampled and frozen in liquid N for biochemical analyses. Prior to pigment extraction, the leaf material was ground to a fine powder in liquid N. Concentrations of flavonoids in tomato leaves were determined with the aluminium chloride method (León-Chan et al., 2017). The concentration of anthocyanins in red cabbage leaves was determined photometrically using the pH shift method after extraction in acidified 80% methanol (Wrolstad et al., 2005). Chlorophylls were extracted from spinach in 100% acetone and measured photometrically according to Ensminger et al. (2001).

For all statistical analyses, Excel (Microsoft Office Standard, 2013) was used. Significant differences of phenols and the Multiplex® index FLAV between tomato plants of the control and N deficiency treatment according to the Student's T-Test were calculated using function TTEST (two-tailed distribution for heteroscedastic samples). Coefficients of determination (R_2) for correlations between Multiplex® indices and biochemical analyses were calculated using linear regression. Pearson product moment correlation coefficient, r, was calculated using the function PEARSON..

3. Results

The experiment with one-month-old tomato plants revealed an impact of N deficiency on flavonoid concentrations in leaves (Fig. 1A) and the Multiplex® index FLAV (Fig. 1B). Flavonoid concentrations in control plants are constantly around 0.14 mg g^{-1} FW^{-1}, while nitrogen-deficient plants show a steady increase up to 0.39 mg g^{-1} FW^{-1} (Fig. 1A). At the same time, we observed a rather constant FLAV index of about 0.07 in control plants, while FLAV of N-deficient plants increases up to 0.18 (Fig. 1B). For both measurements, significant differences are only observed after 7 days.

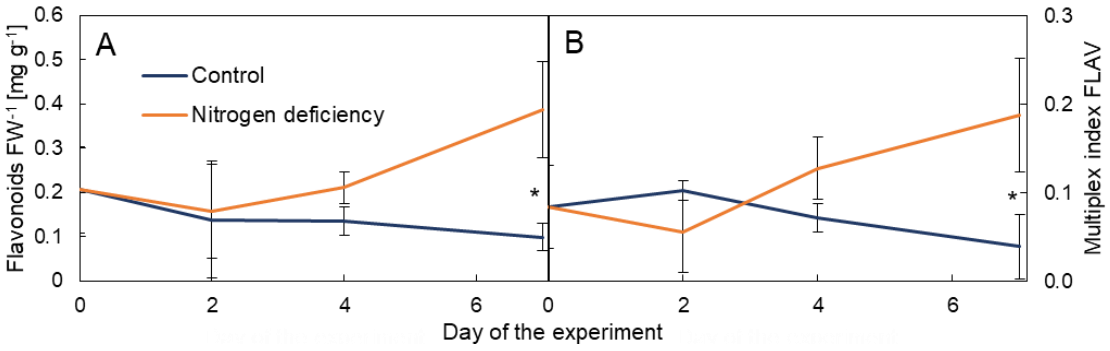

Fig. 1: Total flavonoids and Multiplex® index FLAV in leaves of one-month-old tomato plants exposed to a control and nitrogen deficiency treatment, respectively. n=3±SD; asterisks indicate significant differences with p≤0.05 according to Student's T-Test.

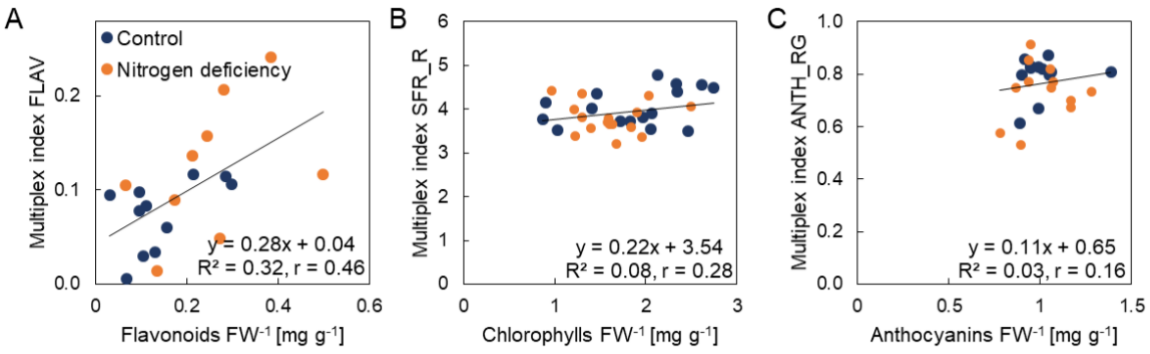

Fig. 2: Correlation between pigment concentrations and the Multiplex® indices. A) Total flavonoids in tomato leaves and the index FLAV, B) Chlorophylls in spinach leaves and the index SFR_R, and C) Anthocyanins in red cabbage leaves and the index ANTH_RG. Each data point represents data from one leaf per plant.

Despite similar trends for total flavonoids and the Multiplex® index FLAV in response to N deficiency (Fig. 1), both parameters show only a weak linear correlation (R_2=0.32; Fig. 2A). No correlations were found for the Multiplex® index SFR_R and the chlorophyll concentrations of spinach leaves (Fig. 2B), as well as the Multiplex® index ANTH_RG and the total anthocyanin concentrations of red cabbage leaves (Fig. 2C).

4. Discussion

Upon exposure to limitations in N supply, plants adjust their metabolic activity, which is visible in altered pigment contents of the leaves. These changes facilitate correlation analysis between multiparametric fluorescence measurements with the Multiplex® and biochemical analyses of flavonoids, anthocyanins and chlorophylls. In the experiment with tomato plants, we observed increasing foliar flavonoid concentrations in N-deficient plants (Fig. 1A), which is a well-known response of tomato plants to abiotic stresses (Løvdal et al., 2010). The Multiplex® index FLAV, representing the flavonoid concentrations, has as well been previously observed to increase in water-deficient and salinity-stressed tomato plants, but this was not confirmed by biochemical analysis (Kautz et al., 2014). Despite a simultaneous increase of the Multiplex® index FLAV (Fig. 1B), we observed only a weak

correlation (R_2=0.32) between total flavonoids and the Multiplex® index FLAV in our N-deficiency experiment (Fig. 2A). However, even weaker correlations between the FLAV index and total flavonoids were found for Asiatic pennywort (*Centella asiatica*) leaves under varying supplies of N (Müller et al., 2013). Higher correlations have been observed for total flavonoids and the FLAV index in lettuce (Zivcak et al., 2017) and kiwifruit exocarps (Pinelli et al., 2013). No correlations between chlorophylls and anthocyanins to the respective Multiplex® indices (Fig. 2B, C) are in contrast to earlier observations of high correlations for chlorophylls in wheat (Tremblay et al., 2012) and anthocyanins in Asiatic pennywort (Müller et al., 2013). This may be related to low variation in pigments in our nitrogen limitation experiments, which was previously also observed in wheat leaves (Peteinatos et al., 2016).

The variability in Multiplex® indices at similar levels of leaf compounds, as seen in Fig. 2, further suggests that these Multiplex® indices may not only be sensitive to variation in the respective class of leaf compounds. Multiplex® indices are calculated based on variation in chlorophyll fluorescence in response to a reference excitation light not absorbed by the leaf compounds and a sampling light specifically absorbed by the leaf compounds of interest, thus estimating their screening effect. Due to a wide range of compounds absorbing light in overlapping wavelength ranges, this method may be prone to varying levels of other leaf compounds (Pfündel et al., 2007). Other factors may have contributed to the observed non-existing or weak correlation between pigment concentrations as assessed by biochemical analyses and by Multiplex® measurements. First, there may be discrepancies in the leaf compounds quantified by biochemical analyses and multiparametric fluorescence measurements using the Multiplex®. Especially the FLAV index is described to specifically quantify the epidermal flavonols, a subset of flavonoids, while the used biochemical method quantifies total flavonoids. Second, Multiplex® indices are most sensitive to epidermal pigments, but biochemical analyses were conducted using whole leaves. Thus, the biochemically derived contents may not reflect the variation in the distribution of flavonoids within the leaf. Higher correlations between Multiplex® and biochemical determination of polyphenols seen in non-leafy products, such as the skin of grapes (Ben Ghozlen et al., 2010) or kiwifruit exocarps (Pinelli et al., 2013) can thus be attributed to higher overlap between measured and sampled plant parts.

5. Conclusions

Many studies rely on multiparametric fluorescence measurements with the Multiplex® to quantify leaf compounds, but only few studies conducted biochemical analyses to confirm measurements with this relatively new and not commonly used device. Our experiments showed that the Multiplex® indicates stress-related changes in leaf metabolism, but also revealed limitations of the Multiplex® to precisely assess pigment concentrations in leafy vegetables. Therefore, we consider the Multiplex® as a useful device to monitor stress-related changes in leaf metabolism over time and to indicate the onset of plant stress responses, but recommend to refrain from using the Multiplex® to quantify leaf compounds without conducting biochemical assays as reference.

6. Literature

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